Con_A-CNT (Carbone Nanotube) conjugate with Short Wave near-Infrared (SWIR) laser ablation for tumor therapy

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ABSTRACT

Using the characteristics of *T cell mitogen* called lectin protein from the jack-beam Canavalia ensiformis *Concanavalin A (Con_A)* with dual activities, *cytotoxicity* and *immunomodulation*, we have shown it has a therapeutic effect on hepatoma. Injection of Con_A can eradicate the established malign tumor, because Con_A can induce tumor cell *autophagic, cell-programmed death*, as well as activate the effector *T cells*. Combined, in this paper, with the absorption exceeding the Carbon NanoTube (CNT) band-gap (ε_{bg}=~1/CNT diameter) with an active short wave near-infrared (SWIR) (1.2~1.5 micron wavelengths), which happened to be translucent to the irradiation upon animal skin, similar to that used in hospital fingertip-clamped Pulse Oxymetry. Once the Con_A-CNT is guided to hepatoma cells, it is bonded and internalized into the ***mitochondria (MC)*** compartment, the cellular energy factory. Con_A has the higher specificity for tumor cells useful for targeting because of the abnormal glycosylation on tumor cells. When CNT hitch hike with Con_A, they can t together like a laser-denotable chemical missile surgically targeting at the tumor cells precisely by Con_A-guidance. We switch on SWIR laser, when the Con_A-CNT conjugated complex has been bonded and internalized to *MC* of malign cells and already commenced cellular programmed death. Thus, it might appear to casual readers that we have initiated an overkill, chemical drugged autophage followed with physical laser ablation, but what if we can eradicate hepatoma totally if no blue print is left behind inadvertently in case of a partial failure. We conclude that using Con_A-CNT conjugated complex targeting specifically at malign tumor cells is a novel targeted-laser-radiation therapy for tumors in mice.

Keywords: Con A, Carbon NanoTube, Hepatoma, Immune Modulation, Thermal Ablation

1. INTRODUCTION

The current radiation treatment for tumor is not fully satisfactory, the major drawback being the fatal relapse after surgery or chemotherapy treatment. Several reasons can explain the deficiency of the tumor therapy, *lacking of tumor- targeting specificity* is the major factor that affects the efficacy of the cytotoxic drug treatment. Relatively speaking, tumor cells is not immunogenic and can not induce effective anti-tumor immunity, instead, immunosuppressive status is established prior to and during tumor growth. And therefore it suppresses the generation of the anti-tumor immunity [1-3]. We have established an *in situ* hepatoma model for the evaluation of any anti-tumor reagent [4]. The transplantable hepatoma cells were injected into the spleen of the syngenic mice, whereupon the

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14. ABSTRACT

Using the characteristics of T cell mitogen called lectin protein from the jack-beam Canavalia ensiformis Concanavalin A (Con_A) with dual activities, cytotoxicity and immunomodulation, we have shown it has a therapeutic effect on hepatoma. Injection of Con_A can eradicate the established malign tumor, because Con_A can induce tumor cell autophagic, cell-programmed death, as well as activate the effector T cells. Combined, in this paper, with the absorption exceeding the Carbon NanoTube (CNT) band-gap (εbg=~1/CNT diameter) with an active short wave near-infrared (SWIR) (1.2~1.5 micron wavelengths) which happened to be translucent to the irradiation upon animal skin, similar to that used in hospital fingertip-clamped Pulse Oxymetry. Once the Con_A-CNT is guided to hepatoma cells, it is bonded and internalized into the mitochondria (MC) compartment, the cellular energy factory. Con_A has the higher specificity for tumor cells useful for targeting because of the abnormal glycosylation on tumor cells. When CNT hitch hike with Con_A, they can t together like a laser-denotable chemical missile surgically targeting at the tumor cells precisely by Con_A-guidance. We switch on SWIR laser, when the Con_ACNT conjugated complex has been bonded and internalized to MC of malign cells and already commenced cellular programmed death. Thus, it might appear to casual readers that we have initiated an overkill, chemical drugged autophage followed with physical laser ablation, but what if we can eradicate hepatoma totally if no blue print is left behind inadvertently in case of a partial failure. We conclude that using Con_A-CNT conjugated complex targeting specifically at malign tumor cells is a novel targetedlaser- radiation therapy for tumors in mice.

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tumor cells first colonized in the spleen and then continue their migration into the liver to form various tumor nodules. The pattern and kinetics of the tumor growth are reasonably predictable, for example, a transplantation of 1×10^6 ML-1 cells will generate tumor nodules in the liver, reaching 150-200 tumor nodules of varying sizes (<1 mm, 1-4 mm, >4 mm in diameter) at three weeks post transplantation. The tumor nodule growth is time-dependent, and the hepatoma-bearing mice will die at around day 40 due to overgrowth of tumors. Many variables can be manipulated, for example, the number of hepatoma cells inoculated can be adjusted for different sizes of tumor nodules or survival of the tumor-bearing mice. The treatment can be commenced at any time depending on the tumor load. Of course, the tumors can also implant on the back so that the tumor growth can be easily handled and measured.

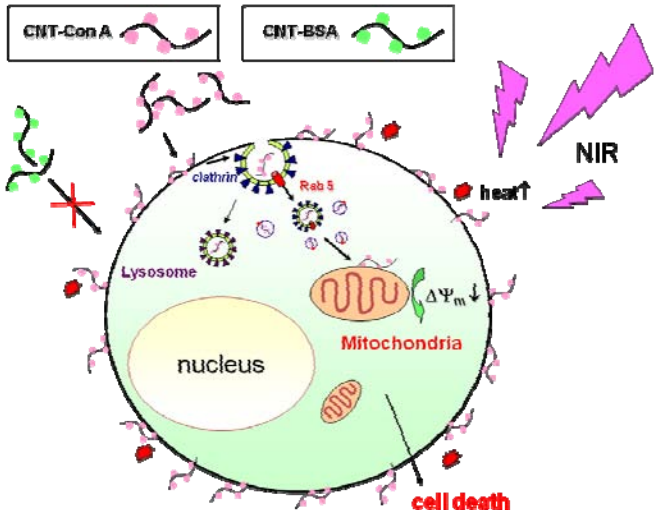

Fig. 1 Schematic diagram of total eradication of hepatoma tumor by double kills using the complex of CNT-CON_A conjugation, denoted in pink. (i) Biochemical drug: Mouse liver malign tumor, hepatoma, is eradicated with the CON_A guided and internalized within a cell commencing through the mitochondria a programmed death. This is furthermore followed by targeted (ii) Physical radiation: we apply targeted thermal ablation initiated by the skin-translucent NIR-laser which matches with the band-gap of the semiconductor Carbon NanoTube(CNT). A placebo control agent is also shown as CNT-BSA in green

Concanavalin A (Con A: a lectin protein from the jack-beam Canavalia ensiformis) is known to be *T* cell mitogen triggering cell division. Con A has been shown to induce hepatitis in (the hamster spleen and) mice through the triggering of natural killer (NK) *T cells* and subsequent activation of helper *positive thymocytes* $CD4^+$ T cells (a class of peptides for professional antigen-presenting (APC) cells of MHC) [5,6]. We have reported that Con A (7.5 mg/kg body weight, twice at three-day interval) administered at one week post transplantation will significantly decrease 150 tumor nodules in the control mice to 40 tumor nodules in the Con A-treated group at 30 days post tumor injection in the *in situ* hepatoma model,. Around 30 – 40% of the mice were tumor-free. In the survival experiment, the survival of the hepatoma-bearing mice was prolonged from 40 to 70 days after Con A treatment, while 20 –30% of the mice were cured. When the dose of Con A and the number of injections were increased, for example to 20 mg/kg and 4 times, the liver tumor nodules

could be completely eradicated. The Con A-activated lymphocytes would infiltrate into the liver to kill the hepatoma cells. The therapeutic effect of Con A can be extended to a large tumor burden. For mice that have hepatoma growth for 2 or 3 weeks, Con A only partially inhibited the tumor nodule growth and prolong the mouse survival. The Con A therapeutic effect decreased along with the increased tumor load. The cells participating in this inhibition were further demonstrated to be $CD8^+$ T as indicated by the *in vivo* depletion of $CD4^+$ or $CD8^+$ T cells. Depletion of $CD8^+$ T cells blocked the anti-tumor effect of Con A whereas depletion of $CD4^+$ T cells also partially affected the Con A anti-tumor activity. Moreover, during the eradication of established hepatoma in the liver, the ML-1 tumor-specific immunities were established and could prevent the next tumor formation. The tumor-free mice after Con A treatment were inoculated subcutaneously with either ML-1 or CT-26 cells dorsally, and the ML-1 cells could no longer grow in ML-1-sensitized mice compared with naïve mice, but CT-26 tumor cells did grow. This suggests that Con A is Con A is a potential anti-tumor agent [7-9]. However, some limitations exist for the broad use of Con A. Con A is a protein and is easily digested after injection into the body, it is immunogenic and will induce antibody response to Con A to inhibit its continuing use of Con A. Readers may skip Fig. 1 for the mutual convenience of immunologists & nanotechnologists.

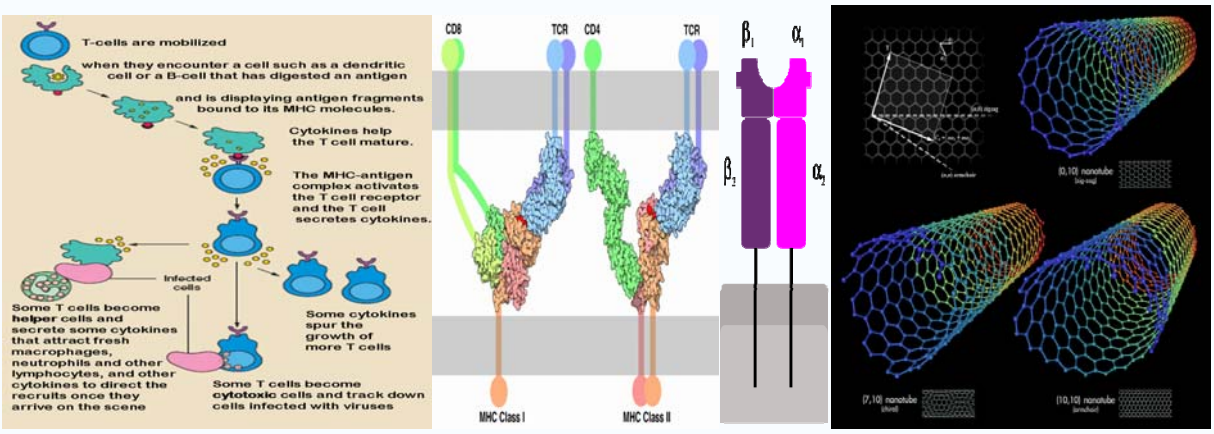

Fig. 2 Immunology 101: White blood cells originate from hematopoietic stem cells in bone marrow during childhood development for "adaptive immune system" in Thymus organ with a variety of "T cells," short for Thymus cells,: so-called helper, cytotoxic, memory, regulator, natural killer (NK), alpha-beta-gamma-delta receptor called T cells receptors (TCR) at MHC (Major Histocompatibility Complex) molecules e.g. macrophages, dendritic cells and B cells*.:*

Nanotechnology 101: Three types of single wall Carbon NanoTubes (CNT) are metal, semi-metal, and semi-conductor, of which the semi-conductor band gap is shown to be inversely proportional to the diameter. One can select a specific diameter of CNT matching at the tissue-translucent Short Wave Infrared (SWIR) light at 1.2 ~1.4 micron wavelength used as the Hospital pulsar Oxymetry for SWIR light backscattering from red vs. blue blood with different oxygen content.

The application of Carbon NanoTubes (CNTs) to biomedical fields is actively investigated because of their useful combination of micro-nano-size, and physics, as well as being conjugated with enzymes for physicochemical properties [10-14]. For cancer research, CNTs can be used for delivering pharmacologic agents, diagnostic imaging agents, DNA, siRNA, oligonucleotides, and

protein to cancer cells. The ability of CNTs to convert rapidly the near-infrared (NIR) light, known as the Short Wave Infrared (SWIR) at 1.2~1.5 micron wavelengths, into heat provides an opportunity to create a new generation of anti-cancer therapy agent. Hyperthermia has been clinically used in the management of tumors because it can synergistically enhance the tumor toxicity when combined with chemotherapy or radiotherapy [15,16]. It can increase the permeability of tumor vasculature compared with normal blood vessels, and result in the enhancement of drug deliver to tumor mass. The use of NIR light in the 700- to 1100-nm range for the hyper-thermal effect is feasible because living tissues do not strongly absorb in this range. However, external NIR light source should effectively and safely penetrate normal tissue and ablate the targeted cells to which CNTs are attached. Therefore, the critical aspect for selective CNT-mediated thermal ablation of cells is to stably deliver and attach the CNT-conjugates to target cells. Several studies have reported the use of mAb or peptide to tumor cells [17-19]. We have reported Concanavalin A (Con A), a plant lectin isolated from *Canavalia ensiformis* (Jack bean) seeds, can agglutinate cells with its mannose-binding specificity and is a novel anti-tumor agents [8,9]. To further extend its application, we combined the Con A to CNTs to use the thermal ablation for tumor cells. The Con A can guide the CTNs to tumor cells specifically, then use NIR light with generate the heat to enhance the anti-tumor effect of Con A.

2. METHODOLOGY

Reagents and cells. Con A, Con A-FITC, BSA, and DAPI were purchased from Sigma (St. Louis, MO). Purified carbon nanotubes were purchased from Carbon Nanotechnologies. Con A was conjugated to carbon nanotubes with Traut's reagent [20]. BALB/c hepatoma cell line, ML-1, was kindly provided by Dr. C.P. Hu (Veterans General Hospital, Taipei, Taiwan). ML-1_{4a} cells were adapted from ML-1 cells in BALB/c mice for four generations.

Microscopic examination. ML-1_{4a}, BHK-21, 3T3 cells were grown in RPMI1640 medium with 10% FBS. To localize the cellular distribution of Con A, ML-1_{4a} cells treated with Con A-FITC were further stained with 1 μg/ml of MitoTracker red or LysoTracker red (Molecular Probe, Oregon, USA), and fixed with 3.7% formaldehyde. In the parallel experiments, the cells treated with Con A-FITC were stained with primary antibody against calnexin followed by secondary antibody conjugated with Alexa 594. The distribution of Con A within cells was analyzed by confocol microscopy (Olympus FV 300, Japan). To observe the autophagic vesicles, the ML-1_{4a} cells were treated with Con A, fixed with 4 % glutaraldehyde and postfixed in 1% OsO_4. The cells were observed under the electron microscopy (Hitachi 7000, Japan).

Ablation of Con A-CNT-FITC binding cells with NIR light. Cells were incubated with Con A-CNT in PBS for 20 min at 4 ^oC. Cells were then washed with ice-cold PBS, and then $1x10^5$ cells dispersed in 96-well plate in 200 μl of medium. The cells were exposed to continuous NIR light by using a FAP-Sys-30W 805- to 811-nm laser system (Coherent) for 7 min at 5 W/cm^2. The cells were observed under fluorescent microscope.

3. RESULTS

CNT-Con A targets to tumor cell and internalized to mitochondria. Con A can bind to the mannose moiety of cell surface glycoproteins specifically because the methyl-αD-mannopyranoside can block this interaction. This binding will induce growth inhibition and cell death in a dose- and time-dependent manner. For tumor cell lines, a high dose is cytotoxic whereas a low dose is cytostatic. The Con A sensitivity varies among different cell lines: the IC_{50} of Con A for HepG2, CT-26, ML-1, and Huh-7 are 5, 10, 10, and 20 μg/ml, respectively. But, for lymphocytes, Con A is a T-cell mitogen at doses of 1-10 μg/ml, while a higher dose is still cytotoxic. Lymphocytes are more sensitive to Con A than tumor cell lines probably due to their high content in mannose- or glucose-containing moiety on the cell membrane. Using the CNT-Con A-FITC conjugate, we found that the bound Con A on the cell membrane will be internalized and accumulated primarily onto the mitochondria as early as 1 h post treatment, and gradually increased mitochondria membrane permeability change (Fig. 1). Some of the endocytosized Con A was then degraded in the lysosome. The increased mitochondria membrane permeability would then lead to the treated cell death with Annexin V-positive, but no DNA ladder or typical apoptosis was observed. Instead, cell death proceeded in an autophagic format. The autophagic pathway characteristics including LC3-II formation, double-layer vesicles, BNIP3 induction, and acidic vesicular organelle formation were observed after Con A treatment. The phosphorylated AKT was also down-regulated, indicating that the growth signal of AKT was altered after Con A treatment. A double-layer vesicle and many autophagosomes were detected on the Con A-treated ML-1 cells. The lysosomal activity as detected by acridine orange staining was increased, and the long stable COX-IV was decreased, indicating the increasing activity of lysosomes. A BNIP3-mediated mitochondria autophagy was induced by Con A. A sustained Con A-induced autophagic flux on tumor cells will lead to cell death.

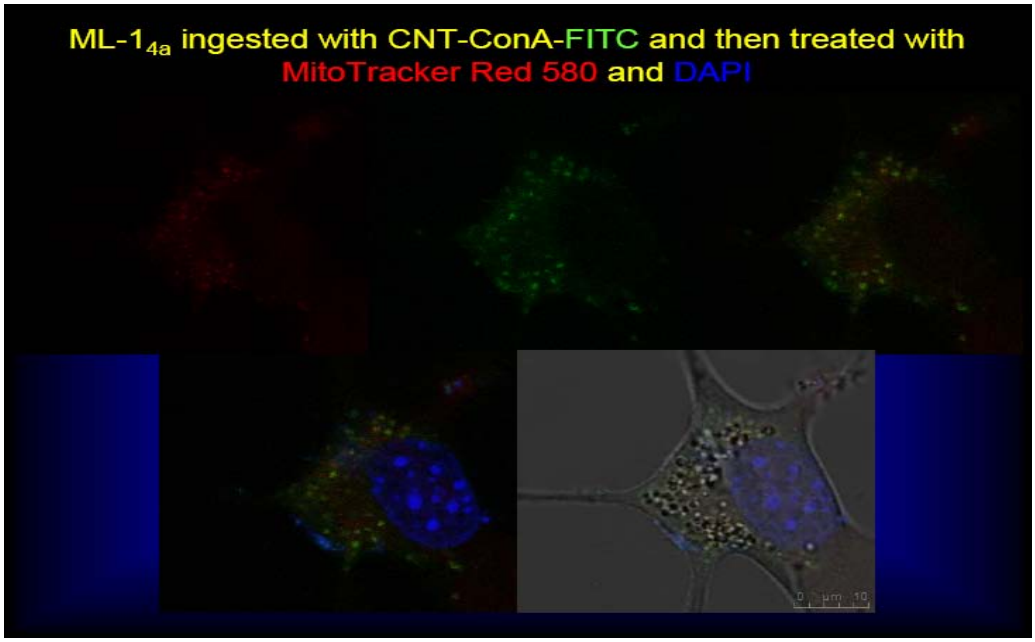

Fig. 1. Con A-CNT conjugate bind to ML-1 cells and internalized into mitochondria.

NIR laser irradiation cause ablation of Con A-CNT bound cells. After binding of Con A-CNTs to tumor cells, the targeted cells were exposed to NIT light. As shown in Fig. 2, the Con A-CNT bound cells were thermally ablated after exposure to NIR light, whereas BSA-CNT control conjugate has no effect. This suggests the specificity of Con A to target the abnormal tumor cells membrane.

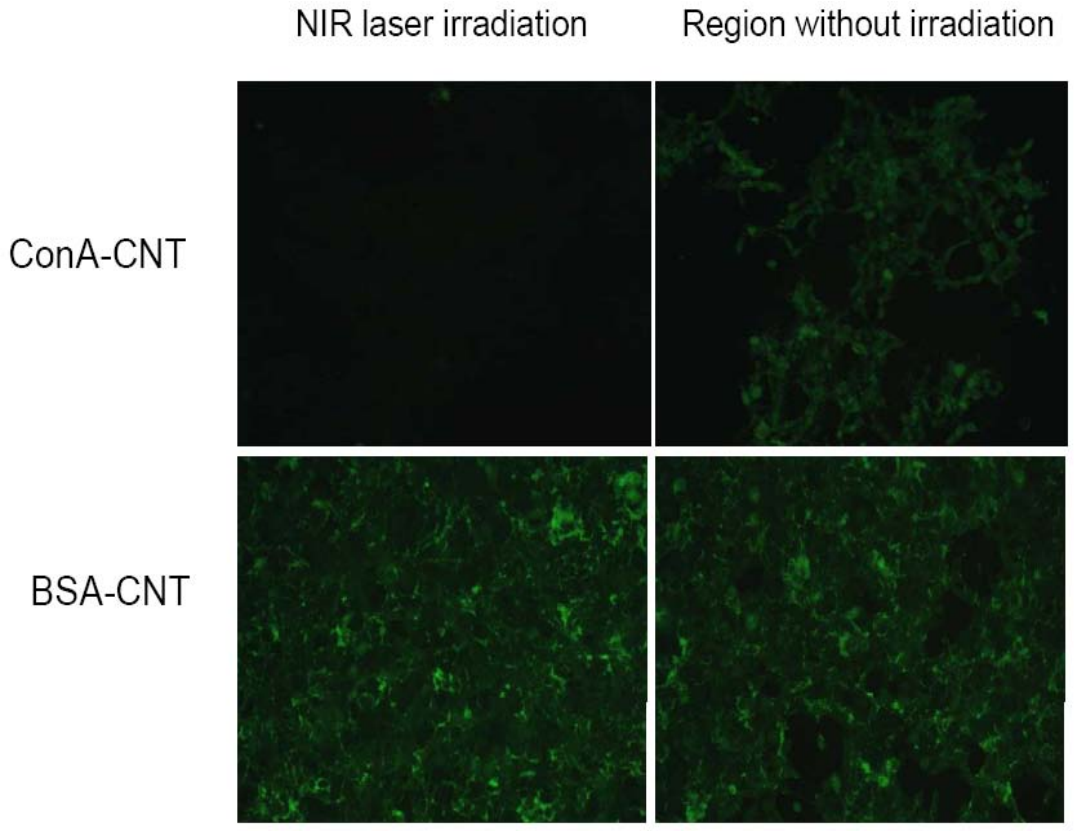

Fig. 2. NIR laser irradiation of tumor cells treated with CNTs conjugated with Con A or BSA.

4. DISCUSSION

How the Con A exerts its anti-tumor effect? Con A trapped in the tumor mass would preferentially bind to tumr cells through its specific binding to the high content of mannose residue on tumor cell membranes. It would not only directly induces tumor cells to undergo autophagic cell death, but also activates lymphocytes into the tumor site. This inflammation subsequently induces the adaptive immune response against the tumor and leads to liver tumor regression. The Kupffer, NK, NKT, $CD4^+$ T, and $CD8^+$ T cells can be activated, but $CD8^+$ T cells are the major effector cells to kill the tumor cells, whereas the $CD4^+$ T cells have an effector function as well as a regulatory function. Initially, the Con A effect is not tumor cell-specific, but as intra-tumor inflammation proceeds following Con A-mediated destruction and activation, tumor antigens will be processed and presented to the tumor antigen-specific $CD4^+$ T and $CD8^+$ T cells. Tumor-specific immunity is

established thereafter. It would be interesting to know how the macrophage or dendritic cells handle the autophagic tumor cells exogenously and present hepatoma cell antigen to $CD4^+$ T and $CD8^+$ T cells in both MHC class II and class I-restricted mechanisms. Con A can stimulate macrophages to up-regulate the TLRs and enhance the cytokine production [21]. The lymphocytes/monocytes are found to be more sensitive to Con A than tumor cells due to their high content of mannose-containing moiety on the cell membrane. Con A at a dose of 1-10 μg/ml is mitogenic, but becomes cytotoxic at dose higher than 40 μg/ml. Furthermore, Con A can activate the NK cells. Miyagi T *et al*. reported that Con A can activate the intrahepatic innate immune cells to provoke an antitumor effect in a NK cell- and IFN-γ-dependent manner in a CT-26 hepatic metastasis model [22].

Kam et al, have reported the functionalized CNT with a folate moiety to target the folate receptor bearing tumor cells, the targeted cells can be selectively eliminated by NIR light [13]. Chakravarty et al. further extend to conjugate antibody to CNT and showed the selectively thermal ablation post NIR light exposure [23]. These studies only use the attached moiety to target the specific cells with ligand or antibody. No further enhancement of the killing is applied. In our study of Con A-CNT, although the in vivo effect of Con A-CNTs needs to be demonstrated, it is reasonable that the attached moiety of Con A has additional effect. It can not only kill the targeted cells, but also activate the immune system to enhance the further killing of tumor cells.

Chemotherapy remains the treatment modality of choice for most advanced cancer while immunotherapy is at the infant stage of cancer treatment. The two are regarded as either unrelated or sometimes antagonistic. However, it has become clear that the chemotherapy-induced tumor cell death process will engage with the anti-tumor immune response, and that the two modalities can be synergistically beneficial for cancer treatment [24-27]. The best strategy of immunotherapy will be the association of both a direct cytotoxic drug effect and an indirect immune-mediated cytotoxic effect. The generation of an immunogenic tumor cell death through the induction of calreticulin, HSP, release of inflammatory mediators, proinflammatory cytokines or HMGB1 will favor the recognition of tumor cell antigens by DC, NK, and T cells. The Con A-induced autophagic cell death with necrosis can initiate a tumor cell-specific immune response, but although Con A can initiate the immune response in a tumor cell in a non-specific manner at the beginning, a tumor-specific immune induction occurs during the eradication of tumor cells, which then leads to a tumor-cell specific response during the later stage. In addition, the thermal effect of Con A-CNTs after NIR light exposure will amplify the direct destruction of tumor cells. Nevertheless, the combination effect of direct autophagic induction on target cells and subsequent immunomodulating activity on lymphocytes via the mannose/glucose binding to tumor cells will induce an *in situ* inflammatory response and the subsequent anti-tumor response.

5. CONCLUSION

The Con A-CNT can bind specifically to tumor cells. With the NIR light exposure, the bound Con A-CNT cells will be thermally ablated. The Con A-CNT provide a novel specific target therapy for tumor.

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